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(54) Title: CHIMERIC PRE–ACTIVATED TRANSCRIPTION FACTORS

(57) Abstract

Disclosed herein is a chimeric protein comprising a pre–activated transcription factor and a strong transcription activation domain for regulating fungal gene expression, and reagents and methods for constructing and using said protein.

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CHIMERIC PRE-ACTIVATED TRANSCRIPTION FACTORS

Background of the Invention

Fungal species are the commercial source of many medicinally useful products, such as antibiotics (e.g., beta-lactam antibiotics such as penicillin, cephalosporin, and their derivatives), anti-hypercholesterolemic agents (e.g., lovastatin and compactin), immunosuppressives (e.g., cyclosporin), and antifungal drugs (e.g., pneumocandin and echinocandin). All of these drugs are fungal secondary metabolites, small secreted molecules that fungi utilize against competitors in their microbial environment. Fungi also produce commercially important enzymes (e.g., cellulases, proteases, and lipases) and other products (e.g., citric acid, gibberellic acid, natural pigments, and flavorings).

The production of secondary metabolites, enzymes, and other products is regulated by coordinated gene expression. For example, the production of penicillin is limited by the activity of two enzymes, encoded by the *ipnA* and *acvA* genes. PacC, a zinc-finger transcription factor, binds to sequences upstream of these two genes. Moreover, increased activity of PacC leads to both increased enzyme activity and penicillin production.

Our understanding of transcriptional regulation of secondary metabolite production, as exemplified above, has increased greatly over the past decade. To date, however, the use of genetically-engineered transcription factors has not been applied to increase production of commercially-important fungal products. In contrast, methods to increase production of penicillin currently rely upon mutagenesis and selection for mutants which display increased secondary metabolite production.

Summary of the Invention

The invention provides a means to increase the production of secondary metabolites in fungi by genetic manipulation of the fungal organism itself. The ability to increase fungal secondary metabolite production has at least two important applications. First, it will allow increased production of existing secondary metabolites which are useful in clinical and experimental settings. Second, increasing production of secondary metabolites will facilitate identification of new compounds in fungi that otherwise make undetectable levels of these compounds in the laboratory.

Accordingly, in one aspect, the invention features a two-part chimeric transcription factor including (i) a pre-activated transcription factor functional in a fungal strain, and (ii) a transcription activation domain that is different from the transcription activation domain naturally associated with the transcription factor. In a preferred embodiment, the transcriptional activity of the chimeric transcription factor is greater than the transcriptional activity naturally associated with the pre-activated transcription factor. In another preferred embodiment, the pre-activated transcription factor is pre-activated by truncation. In a related preferred embodiment, the pre-activated transcription factor includes a substitution of a serine or threonine residue with an alanine, aspartic acid, or glutamic acid residue, wherein the substitution pre-activates the transcription factor (e.g., by mimicking or otherwise altering phosphorylation). In another preferred embodiment, the transcription factor is a member of the PacC family (defined below) and can be pre-activated. In a related preferred embodiment, the pre-activated transcription factor contains portions of the amino acid sequence shown in Fig. 1 (SEQ ID NOs: 1-6).

In another aspect, the invention features a vector including DNA encoding a chimeric transcription factor including (i) a pre-activated

transcription factor functional in a fungal strain, and (ii) a transcription activation domain that is different from the transcription activation domain naturally associated with the transcription factor. The DNA is operably linked to a promoter capable of directing and regulating expression of the chimeric transcription factor in a fungal strain.

The transcription factor encoded within the vector described above is expressed in a fungal cell, such as a filamentous fungal cell, which produces the secondary metabolite of interest and in which expression of the transcription factor increases the production of the secondary metabolite by the cell. The secondary metabolite can be non-proteinaceous or it can be a protein or peptide.

In another aspect, the invention features a method of producing a secondary metabolite of interest, including the steps of (i) introducing into a fungal cell, such as a filamentous fungal cell, a vector including a promoter capable of controlling gene expression in the fungal cell, and a nucleic acid encoding a two-part transcription factor including a DNA-binding domain and a transcription activation domain; and (ii) culturing the fungal cell under secondary metabolite-producing conditions. In a preferred embodiment, the transcription activation domain is different from the transcription activation domain naturally associated with the DNA-binding domain. In other preferred embodiments, the transcription factor is a pre-activated transcription factor (pre-activated by substitution of a serine or threonine residue with an alanine, aspartic acid, or glutamic acid residue, or pre-activated by truncation). In other preferred embodiments, the DNA binding domain of the transcription factor is from a fungal transcriptional activator or from a fungal transcriptional repressor.

By "pre-activated transcription factor" is meant a transcription factor

or fragment thereof that, compared to the precursor molecule, is capable of 1) increased binding, either direct or indirect, to a specific DNA sequence located in a gene regulatory region (e.g., a promoter), or 2) increased transcription activating properties. Pre-activated transcription factors may be able to activate transcription from promoters, but this is not necessarily the case. For example, a transcription factor DNA-binding domain with binding properties but no transactivation activity is considered to be a pre-activated transcription factor. "Pre-activation by truncation" or "pre-activated by truncation" means that removal of a portion of the protein leads to pre-activation. This occurs *in vivo* through proteolytic cleavage. In the invention, pre-activation by truncation is achieved with the use of DNA that encodes a pre-activated form of the protein, excluding portions of the protein that would be proteolytically cleaved *in vivo*.

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 50%, preferably 85%, more preferably 90%, and most preferably 95% identity to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

By "promoter" is meant a sequence sufficient to direct and/or regulate transcription. Also included in the invention are those elements which are sufficient to render promoter-dependent gene expression controllable for cell type-specific, tissue-specific, temporal-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' or intron

sequence regions of the native gene.

By "operably linked" is meant that a gene and one or more regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequences.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Drawing

Fig. 1 is an alignment of the zinc-finger DNA-binding domain of PacC family members from *Aspergillus nidulans* (SEQ ID NO: 1), *Aspergillus niger* (SEQ ID NO: 2), *Penicillium chrysogenum* (SEQ ID NO: 3), *Yarrowia lipolytica* (SEQ ID NO: 4), *Candida albicans* (SEQ ID NO: 5), and *Saccharomyces cerevisiae* (SEQ ID NO: 6). Identity is represented by shaded regions; similarity is represented by boxed regions.

Detailed Description

The invention features a two-part chimeric protein including a pre-activated transcription factor and a strong transcription activation domain for regulating fungal gene expression. The protein is encoded by nucleic acids operably linked to a strong promoter in a vector which allows for expression in fungal cells. The effect of the transcription factor is to facilitate expression of a protein which itself is a desired product, or which acts as an element (e.g., an enzyme) by which a desired product is made by the host fungal cell. Each of these components is described below. Experimental examples described herein are intended to illustrate, not limit, the scope of the claimed invention.

Pre-Activated Transcription Factor

The vectors of the invention can include DNA encoding any proteinaceous transcription factor that can be provided in pre-activated form; i.e., the vector encodes the protein in a form in which it is already activated; i.e., no post-translational processing is required for the protein to be active in a fungal cell to bind to regulatory DNA of the cell to facilitate gene expression.

Transcription factors regulate the level of gene expression by affecting the activity of the core transcriptional machinery at the promoter of each gene. Several mechanisms have evolved to control the activity of transcription factors.

Post-translational modification is one mechanism by which transcription factors are regulated. Proteolytic cleavage is one post-translational mechanism for regulating the activity of a transcription factor (e.g., Pahl and Baeuerle, *Curr. Opin. Cell Biol.*, 1996, 8:340-347; Goodbourn and King, *Biochem. Soc. Trans.*, 1997, 25:498-502; Fan and Maniatis, *Nature*, 1991, 354:395-398). The fungal PacC family of transcription factors is one class of proteins that can be activated by proteolysis. Activating mutations have been described for PacC family members (see below); these mutations truncate the encoded protein, resulting in the production of a pre-activated form of the transcription factor.

Another method for pre-activating a transcription factor is to mimic the modifications which normally regulate its activity. For example, phosphorylation has been shown to positively regulate the activity of some transcription factors and negatively regulate that of others (see review by Hunter and Karin, *Cell*, 1992, 70:375-387). Other forms of post-translational modifications that can increase the activity of transcription factors include acetylation (Gu and Roeder, *Cell*, 1997, 90:595-606) and alkylation (e.g.,

methylation)(Chinenov et al., *J. Biol. Chem.*, 1998, 273:6203-6209; Sakashita et al., *J Biochem (Tokyo)*, 1995, 118:1184-1191).

Dephosphorylation of particular residues can increase the activity of many transcription factors. Phosphorylation most commonly occurs on serine (Ser), threonine (Thr), and tyrosine (Tyr) residues; in some instance residues such as aspartate (Asp) and histidine (His) can be phosphorylated. The coding sequence for the phosphorylated residue can be mutated to encode an amino acid that cannot be phosphorylated and does not have a negatively charged side chain (e.g., alanine (Ala)). Ser→Ala, Thr→Ala, Tyr→Ala, and Asp→Ala substitutions are frequently used in the art to produce a pre-activated transcription factor (see, for example, Chen et al., *Proc. Natl. Acad. Sci. U.S.A.*, 1998, 95:2349-2354; Song et al., *Mol. Cell Biol.*, 1998, 18:4994-4999; O'Reilly et al., *EMBO J.*, 1997, 16:2420-2430; Hao et al., *J. Biol. Chem.*, 1996, 271:29380-29385).

Phosphorylation can also increase the activity of a transcription factor. Mutations of Glu or Asp for Ser, Thr, or Tyr are frequently used in the art to mimic a phosphorylation event and pre-activate a transcription factor (see, for example, Hoeffler et al., *Nucleic Acids Res.*, 1994, 22:1305-12; Hao et al., *supra*). Mutations that result in a substitution of Glu for Asp, at Asp residues which can be phosphorylated, can also cause activation (Klose et al., *J. Mol. Biol.*, 1993, 232:67-78; Krems et al., *Curr. Genet.*, 1996, 29:327-34; Nohaile et al., *J. Mol. Biol.*, 1997, 273:299-316).

Other mutations can be made that mimic activating post-translational modifications. For example, the *E. coli* Ada transcription factor is activated by methylation of cysteine (Cys) residue 69. A Cys→His substitution was found to result in activation (Taketomi et al., *Mol. Gen. Genet.*, 1996, 250:523-532). This particular substitution was identified by substituting Cys 69 with each of

the other nineteen amino acids. Alternatively, in instances where no obvious substitution can be made to mimic a modification (e.g., acetylation), a random mutagenesis is performed to identify constitutively active forms of transcription factors (see, for example, Onishi et al., *Mol. Cell Biol.*, 1998, 18:3871-3879). This technique can employ simple and rapid phenotypic or reporter selections, such as those described herein, to identify activated forms. For example, a *Saccharomyces cerevisiae* strain containing a reporter construct can be used to select for activated forms Specifically, the *ipnA* promoter (P_{ipnA}) from *Aspergillus nidulans* may be fused to a gene from *Saccharomyces cerevisiae* that confers a growth advantage, such as *HIS3*, when PacC is pre-activated by a mutation. A P_{ipnA}-*HIS3* fusion has the added advantage that expression levels can be titrated by the compound 3-aminotriazole (3-AT). 3-AT is a competitive inhibitor of His3 that, when present in sufficient amounts, will inhibit the His3 expressed from P_{ipnA} and prevent this strain from growing on SC-HIS. In this example, *pacC* coding sequence can be randomly mutagenized and vectors containing the mutated alleles are transformed into the reporter strain. Growth of a strain containing P_{ipnA}-*HIS3* only occurs on SC-HIS+3-AT plates when P_{ipnA}-*HIS3* expression is increased to overcome the competitive inhibition of His3 by 3-AT. This method provides a rapid technique for screening for mutations which pre-activate a transcription factor.

The PacC Family of Transcription Factors

One group of transcription factors useful in the invention are members of the PacC family. The PacC transcription factors regulate gene expression in response to changes in ambient pH. Members of the family have the following characteristics: 1) They display significant (at least 35%) amino acid sequence identity to the *Aspergillus nidulans* PacC protein (Tilburn et al.,

EMBO J., 1995, 14:779-790). Such proteins have been identified in *Yarrowia lipolytica* (YlRim101p; Lambert et al., *Mol. Cell. Biol.*, 1997, 17:3966-3976), *Penicillium chrysogenum* (Suarez and Penalva, *Mol. Microbiol.*, 1996, 20:529-540), *Aspergillus niger* (MacCabe et al., *Mol. Gen. Genet.*, 1996, 250:367-374), *Saccharomyces cerevisiae* (Inv8/Rim101/Rim1; Su and Mitchell, *Nucleic Acids Res.*, 1993, 21:3789-3797), and *Candida albicans* (U.S.S.N. __/_____)(Table 1). 2) They contain a predicted DNA-binding region that includes three zinc fingers of the Cys_2His_2 class.

TABLE 1

Species of origin of PacC homolog	% identity to *A. nidulans* PacC in 107-aa	% similarity to *A. nidulans* PacC over entire length
A. Niger	94	75
P. chrysogenum	84	67
C. albicans	61	18
S. cerevisiae	56	22
Y. lipolytica	58	30

In addition, several PacC family member either have been shown to directly bind to or regulate expression of genes that contain a 5'-GCCAAG-3' or 5'-GCCAGG-3' element in upstream regulatory sequence (Tilburn et al., *supra*; Suarez and Penalva, *supra*). Furthermore, with the exception of PacC from *P. chrysogenum*, mutations that truncate the protein have either been identified or constructed, and these mutations result in activation of gene expression by the PacC family of proteins, even at low ambient pH (Tilburn et al., *supra*; van den Hombergh et al., *Mol. Gen. Genet.*, 1996, 251:542-550; Lambert et al., *supra*; Li and Mitchell, *Genetics*, 1997, 145:63-73). Finally, in both *A. nidulans* and *S. cerevisiae*, it has been demonstrated

that specific proteolytic cleavage results in activation of signaling *in vivo* (Orejas et al., *Genes Dev.*, 1995, 9:1622-32; Li and Mitchell, *supra*).

Transcription Activation Domains

Transcription activation domains (TADs) are discrete regions of proteins which promote gene expression by a variety of mechanisms that ultimately result in the activation of RNA polymerase. A TAD generally is defined as the minimal motif that activates transcription when fused to a DNA-binding domain (DBD) (Webster et al., *Cell*, 1988, 52:169-178; Fischer et al., *Nature*, 1988, 332:853-856; Hope et al., *Nature*, 1988, 333:635-640). The invention can employ any TAD that can transactivate expression from a fungal gene promoter when the TAD is fused to an appropriate DBD. TADs are classified based on similarities in protein sequence and/or composition properties. These classes include the acidic-rich (e.g., Gal4, Gcn4, VP16, and Jun; Webster et al., *supra*; Fischer et al., *supra*; Hope et al., *supra*; Cress and Triezenberg, *Science*, 1991, 251:87-90; Struhl, *Nature*, 1988, 332:649-650), glutamine-rich (Sp1, Oct1, and Oct2; Courey and Tjian, Cell, 1988, 55:887-898; Tanaka et al., *Mol. Cell Biol.*, 1994, 14:6046-6055; Tanaka and Herr, *Mol. Cell Biol.*, 1994, 14:6056-6067), and proline-rich TADs (CTF, NF-I, and EKLF; Mermod et al., *Cell*, 1989, 58:741-753; Tanese et al., *Genes Dev.*, 1991, 5:2212-2224; Chen and Bieker, *EMBO J.*, 1996, 15:5888-5896). Any of these classes of TADs may be used in the present invention. The ability of any particular TAD to transactivate from a particular promoter can be determined using simple, known selection screens.

It is also possible to artificially create either a TAD or a site-specific DBD. In one example, protein sequences which transactivate a reporter gene from a promoter of interest are selected from an expression library. In another example, protein sequences which specifically bind particular DNA sequences are selected. In each case, these sequences can then be mutated in a reiterative process to obtain either the optimal TAD sequence for the particular promoter, or the optimal DBD sequence for a particular DNA sequence. Transcription factors containing artificial elements

produced by this or any other method are useful in the invention.

In the chimeric transcription factor of the featured invention, TADs may be used alone or in combination. For example, Sp1 contains multiple glutamine-rich TADs, and these domains act synergistically to promote gene expression (Courey and Tjian, *supra*; Courey et al., *Cell*, 1989, 59:827-836). Oct-2 contains both glutamine-rich and proline-rich TADs, and both are required for maximal expression when fused to either the Oct-2 or a heterologous DBD (Tanaka et al., *supra*). Thus, the use of two or more classes of TADs in one construct may amplify the induction of expression. Furthermore, homopolymeric stretches of proline or glutamine function as TADs (Gerber et al., *Science*, 1994, 263:808-811). In one example, a strong transcription factor has been created by fusion of the Gal4 DBD to a homopolymeric glutamine stretch linked to reiterated VP16 TADs (Schwechheimer et al., *Plant Mol. Biol.*, 1998, 36:195-204).

Fungal Promoters

The chimeric, pre-activated transcription factor is operably linked to a strong promoter, allowing for expression of the transcription factor in a fungal cell. Expression systems utilizing a wide variety of promoters in many fungi are known, including, but not limited to, *Aspergillus nidulans* (*gpd*: Punt et al., *Gene*, 1987, 56:117-124; Hunter et al., *Curr. Genet.*, 1992, 22:377-383; Glumoff et al., *Gene*, 1989, 84:311-318. *alcA*; Fernandez-Abalos et al., *Mol. Microbiol.*, 1998, 27:121-130. *glaA*: Carrez et al., *Gene*, 1990, 94:147-154. *amdS*: Turnbull et al., *Appl. Environ. Microbiol.*, 1990, 56:2847-2852), *Aspergillus niger* (*gpd*: Punt et al., *supra*; Hunter et al., *supra*; Glumoff et al., *supra*. *glaA*: Tang et al., Chin. J. Biotechnol., 1996, 12:131-136. *amdS* promoter: Turnbull et al., *supra*), *Pichia pastoris* (alcohol oxidase I promoter: Payne et al., *Gene*, 1988, 62:127-134), *Pleurotus ostreatus* (*Lentinus edodes ras* promoter: Yanai et al., *Biosci. Biotechnol. Biochem.*, 1996, 60:472-475), *Phytophthora infestans* (*Bremia lactucae Hsp70*: Judelson et al., *Mol. Plant Microbe Interact.*, 1991, 4:602-607), *Neurospora crassa* (*his3* promoter: Avalos et al., *Curr.*

Genet., 1989, 16:369-372), *Yarrowia lipolytica* (*XPR2* promoter: Nicaud et al., *Curr. Genet.*, 1989, 16:253-260. *TEF*: Muller et al., *Yeast*, 1998, 14:1267-1283.), *Penicillium chrysogenum* (*phoA* promoter: Graessle et al., *Appl. Environ. Microbiol.*, 1997, 63:753-756), *Rhizopus delemar* (*pyr4* promoter: Horiuchi et al., *Curr. Genet.*, 1995, 27:472-478), *Gliocladium virens* (*prom1*: Dave et al., *Appl. Microbiol. Biotechnol.*, 1994, 41:352-358), and *Cochliobolus heterostrophus* (Monke and Shafer, *Mol. Gen. Genet.*, 1993, 241:73-80).

There are also simple techniques for isolating promoters in organisms with relatively unstudied genetics. One of these is a system based on selection of sequences with promoter activity (see, for example, Turgeon et al., *Mol. Cell Biol.*, 1987, 7:3297-3305; Weltring, *Curr. Genet.*, 1995, 28:190-196). This approach provides an easy method for isolating promoter fragments from a wide variety of fungi.

The constructs of the invention also preferably include a terminator sequence located 3' to the chimeric transcription factor coding sequence. Terminator sequences which function in numerous fungi are known in the art. These include those from *Aspergillus nidulans trpC* (Punt et al., *supra*; Hunter et al., *supra*; Glumoff et al., *supra*), *Lentinus edodes priA* (Yanai et al., *supra*), *Bremia lactucae Ham34* (Judelson et al., *supra*), and *Aspergillus nidulans argB* (Carrez et al., *supra*).

Construction of Chimeric Transcription Factors

The pre-activated transcription factors of the invention display 1) increased binding, either direct or indirect, to a specific DNA sequence located in a gene regulatory region (e.g., a promoter) *in vivo*, and/or 2) increased transcription activating properties, relative to the precursor molecule. To this end, it is preferable that part or all of the DBD, the domain of the parental transcription factor which recognizes and binds to the DNA sequences, remain intact. Additional sequences from the parental transcription factor may also remain in the chimeric construct, or they may be removed. The TAD of the

parental transcription factor may be removed, as the chimeric transcription factor will contain a TAD from another protein, such as the herpesvirus transactivator VP16, as described herein. The TAD from the parental transcription factor may also remain in the chimeric construct.

As described above, TADs can be acidic, glutamine-rich, or proline-rich. The ability of each of these TADs to function in any given fungal strain will vary. The acidic TADs have been shown to function in a wide variety of organisms, from *C. elegans* to humans, including fungi. Glutamine-rich and proline-rich TADs have also been shown to function in disparate organisms, including fungi. As described above, increased transactivation activity may be achieved by using multiple TADs from one category (Tanaka and Herr, *supra*). Furthermore, TADs from more than one class may be used in one chimeric protein (Schwechheimer et al., *supra*; Tanaka et al., *supra*). In the example described below, 4 VP16 TADs and a proline-rich TAD are placed in series.

The production of chimeric transcription factors which activate transcription is not limited to the use of parental transcription factors that themselves are transcriptional activators. Using this method, transcription factors which are transcriptional repressors may be converted to transcriptional activators by the addition of a TAD. An example is the *Saccharomyces cerevisiae* Mig1, which is a repressor of *SUC2* expression. Deletion of *mig1* derepresses *SUC2* expression. A chimeric protein in which the DBD of Mig1 is fused to the VP16 TAD can activate transcription from promoters containing Mig1-binding sites and leads to increased expression of *SUC2* (Ostling et al., *Mol Cell Biol.*, 1996, 16:753-61). Thus, the formation of a chimeric transcriptional activator may be performed for any transcription factor, whether it be an activator or a repressor.

The choice of parental transcription factor for use in the present

invention depends upon the desired product one wishes to produce. The transcription factor must recognize a sequence in the promoter of a gene of interest. This gene may encode a protein which itself is a desired product, or one which acts as an element (e.g., an enzyme) in the pathway by which a desired product is made by the host fungal cell. For example, a chimeric transcription factor including PacC may be used if the desire is to increase the production of beta-lactam antibiotics. This is achieved by increasing the expression of at least two genes, *ipnA* and *acvA*, which encode enzymes in the penicillin production process.

One skilled in the art will recognize that there are standard techniques, including the ones described herein, which allow for rapid selection and screening of chimeric transcription factor constructs in order to ascertain which transcription factors are the strongest transcriptional activators.

Construction of Fungal Expression Vectors

To achieve high expression of the chimeric transcription factor, several types of expression vectors are known in the art (e.g., those described herein). The choice of expression vectors may depend on the type of fungus to be used. For example, expression of a chimeric transcription factor in *Aspergillus nidulans* may be achieved using the *amdS* promoter system (Turnbull et al., *supra*). The promoter element may be modified such that it also contains a DNA sequence recognized by the chimeric transcription factor. The expression of the chimeric transcription factor will induce increased activation from its own promoter, thus amplifying its own production. The expression vector may also include terminator sequences, as described above. For example, a suitable terminator for *Aspergillus nidulans* is the *argB* terminator.

The vector, once transformed into a fungal cell as described herein, may remain episomal, in which case the vector may also have an origin of replication. The vector may also integrate into the chromosomal DNA of the host cell. The expression of the integrated expression construct may depend on positional effects, and, thus, it may be necessary to screen through or select for transformants to isolate those with suitably high expression. Methods for screening and selection are described herein. The integrated expression construct may also alter the expression of endogenous genes of the fungal cell. This altered expression may be beneficial or detrimental to the survival of the cell or to the purpose of the production of the fungal cell. For example, if the purpose is to increase production of a beta-lactam antibiotic, then loss of expression of *ipnA* (which encodes isopenicillin N-synthase and is required for beta-lactam production) following integration of the expression construct would negate any benefits resulting from expression of the chimeric transcription factor. Thus, a secondary screen of transformants displaying characteristics suitably for the designed purpose may be performed. Methods for determining metabolite production are described herein.

In some cases, it may be beneficial to use a transcription factor which is not chimeric. Overexpression of a parental transcription factor may lead to an increase in secondary metabolites. This overexpressed protein may be constitutively active, due to overexpression or genetic mutation, or it may be regulated in a manner similar to the endogenous transcription factor. The fungal cell may be a wild-type strain, or it may contain one or more mutations (which may also increase production of secondary metabolites). Example mutations include those which result in duplication or rearrangement of biosynthetic genes (e.g., the penicillin gene cluster of *ipnA*, *acvA*, and *aatA*). Reporter genes, such as those described herein, or other exogenous genes may

also be present in the fungal cells, either episomally or chromosomally.

Transformation

In order to introduce the construct into a fungal cell, one may utilize any of numerous transformation protocols (for review, see Punt and van den Hondel, *Methods Enzymol.*, 1992, 216:447-457; Timberlake and Marshall, *Science*, 1989, 244:1313-1317; Fincham, *Microbiol. Rev.*, 1989, 53:148-170). Suitable DNA transformation techniques include electroporation, polyethylene glycol-mediated, lithium acetate-mediated, and biolistic transformation (Brown et al., *Mol. Gen. Genet.*, 1998, 259:327-335; Zapanta et al., *Appl. Environ. Microbiol.*, 1998; 64:2624-2629; Thompson et al., *Yeast*, 1998, 14:565-571; Barreto et al., *FEMS Microbiol. Lett.*, 1997, 156:95-99; Nicolaisen and Geisen, *Microbiol. Res.*, 1996, 151:281-284; Wada et al., *Appl. Microbiol. Biotechnol.*, 1996, 45:652-657; Ozeki et al., *Biosci. Biotechnol. Biochem.*, 1994, 58:2224-2227; Lorito et al., *Curr. Genet.*, 1993, 24:349-356; Oda and Tonomura, *Curr. Genet.*, 1995, 27:131-134). If desired, one may target the DNA construct to a particular locus. Targeting homologous recombination techniques are currently practiced in many fungi, including, but not limited to, *Candida albicans* (Fonzi and Irwin, *Genetics*, 1993, 134: 717-728), *Ustilago maydis* (Fotheringham and Hollman, *Mol. Cell Biol.*, 1989, 9:4052-4055; Bolker et al., *Mol. Gen. Genet.*, 1995, 248:547-552), *Yarrowia lipolytica* (Neuveglise et al., *Gene* 1998, 213:37-46; Chen et al., *Appl. Microbiol. Biotechnol.*, 1997, 48:232-235; Cordero et al., *Appl. Microbiol. Biotechnol.*, 1996, 46:143-148), *Acremonium chrysogenum* (Skatrud et al., *Curr. Genet.*, 1987, 12:337-348; Walz and Kuck, *Curr. Genet.*, 1993, 24:421-427), *Magnaporthe grisea* (Sweigard et al., *Mol. Gen. Genet.*, 1992, 232:183-190); Kershaw et al., *EMBO J.*, 1998, 17:3838-3849), *Histoplasma capsulatum* (Woods et al., *J. Bacteriol.*, 1998, 180:5135-5143)

and *Aspergillus sp.* (Miller et al., *Mol. Cell Biol.*, 1985, 5:1714-1721; de Ruiter-Jacobs et al., *Curr. Genet.*, 1989, 16:159-163; Gouka et al., *Curr. Genet.*, 1995, 27:536-540; van den Hombergh et al., *Mol. Gen. Genet.*, 1996, 251:542-550; D'Enfert, *Curr. Genet.*, 1996, 30:76-82; Weidner et al., *Curr. Genet.*, 1998, 33:378-385).

Methods for Selection and Screening Transformants

Reporter genes are useful for isolating transformants expressing functional chimeric transcription factors. The reporter genes may be operably linked to promoter sequence which is regulated by the chimeric transcription factor. Reporter genes include, but are not limited to, genes encoding β-galactosidase (*lacZ*), β-glucoronidase (*GUS*), β-glucosidase, and invertase, amino acid biosynthetic genes, e.g., the yeast *LEU2*, *HIS3*, *LYS2*, *TRP1* genes (or homologous genes from other fungi, such as filamentous fungi, that encode proteins with the similar functional activities), nucleic acid biosynthetic genes, e.g., the yeast *URA3* and *ADE2* genes (or homologous genes from other fungi, such as filamentous fungi, that encode proteins with the similar functional activities), the mammalian chloramphenicol transacetylase (CAT) gene, or any surface antigen gene for which specific antibodies are available. A reporter gene may encode a protein detectable by luminescence or fluorescence, such as green fluorescent protein (GFP). Reporter genes may encode also any protein that provides a phenotypic marker, for example, a protein that is necessary for cell growth or viability, or a toxic protein leading to cell death, or the reporter gene may encode a protein detectable by a color assay leading to the presence or absence of color.

The choice of reporter gene will depend on the type of fungal cell to be transformed. It is preferable to have two reporter genes within the fungal

cell. One reporter gene, when expressed, may provide a growth advantage to transformed cells which are expressing the chimeric transcription factor. This allows for isolation of such transformants though selective pressures. The other reporter gene may provide a colorimetric marker, such as the *lacZ* gene and its encoded protein, β-galactosidase. Alternatively, the second reporter may provide a fluorescent or luminescent marker, such as GFP. These reporters provide a method of quantifying expression levels from expression constructs comprising chimeric transcription factors. Screens and selections similar to the ones described may be used to optimize construction of chimeric transcription factors or expression constructs.

Example

The following example describes a method for increasing the level of PacC activity over that caused by proteolysis or specific truncations. This invention may facilitate the increased production of fungal secondary metabolites including, but not limited to, penicillins and cephalosporins. Similar genetic engineering can be performed to alter the function of other transcription factors.

A construct that encodes a chimeric transcription factor is described below. In this example, a proline-rich TAD followed by multiple copies of the acidic-rich TAD from the herpes simplex virus VP16 protein are fused to a truncated, pre-activated PacC from *Aspergillus nidulans* (SEQ ID NO: 7). This construct may be integrated at the *pyrG* locus in *Aspergillus nidulans,* as described below. Expression of this chimeric polypeptide is regulated by the strong PGK promoter from *Aspergillus nidulans* and terminator sequences from the *crnA* gene of *Aspergillus nidulans*.

Several DNA cloning steps are required to create this chimeric construct. Bluescript KS (Stratagene, LaJolla,CA) is be used as a cloning vector. The primers 5'- aactgcagTAGTTGACCGTGTGATTGGGTTCT -3' (SEQ ID NO: 8)(lowercase letters denote sequences introduced for cloning and restriction sites are underlined) and 5'- ccggaattcTTTGTAAACTGGCTTGAAGAT -3' (SEQ ID NO: 9) are used to amplify 347bp of *crnA* terminator sequence from genomic DNA template. The PCR product is *Pst*I/*Eco*RI digested and then cloned into the KS polylinker to produce p1. Subsequently, complementary oligonucleotides 5' - gatccCCCCCCCCTCCTCCACCCCCACCCCCTCCC -3' (SEQ ID NO: 10) and 5'- GGGAGGGGGTGGGGGTGGAGGAGGGGGGGGg-3' (SEQ ID NO: 11) are annealed (this double-stranded oligonucleotide encodes a proline-rich motif) and the double-stranded product is ligated into *Sma*I/*Bam*HI digested p1, yielding p2.

Next, the oligonucleotide primers 5'- cgcgatatcAAAGTCGCCCCCCCGACCGAT -3' (SEQ ID NO: 12) and 5'- cgcgatatcCCCACCGTACTCGTCAATTCC -3' (SEQ ID NO: 13) are used in PCR reactions to amplify a 258bp fragment using pVP16 (Clontech, Palo Alto, CA) as template. This product encodes the acidic-rich domain of VP16. The product is digested with *Eco*RV, and ligation reaction is performed with >20 fold excess of *Eco*RV insert relative to *Sma*I-digested calf-alkaline phosphatase treated p2. Bacterial transformants are screened for plasmids that contain multiple tandem insertions of VP16 sequence. *Sma*I sites within the VP16 coding sequence allow for determination of the orientation of the insertion. Plasmids are selected that contain four insertions of the VP16 acidic-rich domain (p3). p3, then, encodes a proline-rich domain in-frame with four

reiterations of the VP16 domain, and these TADs are linked to the *crnA* terminator.

In the next cloning step a truncated form of *pacC* is fused to the coding sequence for the TADs. Primers 5 '-
tgctctagaGGCGCCATGGCCGAAGAAGCG -3' (SEQ ID NO: 14) and 5'-cgcggatccGTAACCAGAAGTCATACCGTC -3' (SEQ ID NO: 15) are used to amplify a 1419bp product (SEQ ID NO: 16) from an *Aspergillus nidulans* cDNA library. This product is *XbaI/BamHI* digested and ligated into digested p3 to produce p4. This cloning reaction introduces a form of *pacC* that lacks the carboxy-terminal 209 amino acids in-frame with the described TADs.

An additional cloning step is required in order to place the coding sequence for this chimera under the control of a strong promoter. Primers 5'-ataagaatgcggccgcCCTCTGCATTATTGTCTTATC -3' (SEQ ID NO: 17) and 5'- tgctctagaAGACATTGTTGCTATAGCTGT -3' (SEQ ID NO: 18) are used to amplify 689bp of PGK promoter sequence (SEQ ID NO: 19) from *Aspergillus nidulans* genomic DNA. This fragment is *NotI/XbaI* digested and cloned into digested p4 in order to yield p5. Thus, p5 contains coding sequence for an 815 amino acid chimeric transcription factor to be expressed from the PGK promoter.

To decrease the extent of position effects, the p5 construct is targeted to the *pyrG* locus. Oligonucleotides 5'-tccccgcggATGGAAGCTTCGTTAAGGATAATT-3' (SEQ ID NO: 20) and 5'-ataagaatgcggccgcCTACCAGATTAGGGAGCATAT-3' (SEQ ID NO: 21) are used to amplify a 2240bp product (SEQ ID NO: 22) from *Aspergillus nidulans* genomic DNA; this product contains coding and regulatory sequence for the *pyrG* gene that encodes orotidine-5'- phosphate decarboxylase. The 2240bp fragment is *SacII/NotI* digested, and then cloned into p5 to produce p6; this

fragment is also cloned into KS to yield p7 (a control construct, containing regulatory sequence for the *pyrG* gene, but no PGK promoter or transcription factor). p6 and p7 are vector that can complement uridine auxotrophy, allowing for selection, and target the chimeric transcription factor to the *pyrG* locus. In addition, primers 5'- tgctctagaGGCGCCATGGCCGAAGAAGCG -3 (SEQ ID NO: 23) and 5' tccccgggGTAACCAGAAGTCATACCGTC -3' (SEQ ID NO: 24) are used to amplify the truncated form of PacC from an *Aspergillus nidulans* cDNA library . This fragment can be cloned into *Xba*I/*Sma*I digested p6 to produce p8. p8 is a control construct, used to monitor the activity of pre-activated PacC expressed from the PGK promoter, independent of the presence of heterologous TADs.

PEG-$CaCl_2$ (or other methods, described herein) may be used to transform protoplasts of a uridine auxotroph carrying a *pyrG* mutation (Ballance and Turner, *Gene*, 1985, 36:321-331). p6, p7, and p8 plasmid DNA are used to transform to uridine prototrophy. PCR and Southern analysis are performed to confirm single-copy integration at *pyrG*.

Several methods may be employed to assess the activity of wild-type, pre-activated, and chimeric PacC-TAD factors. Samples of mycelia may be taken from parallel fermentation of strains containing p6, p7, and p8. Northern blot analysis may be performed on RNA prepared from extracts of these mycelia. Probes are prepared from coding sequence for the *ipnA* and *acvA* genes of *Aspergillus nidulans*. Reporter constructs are valuable tools for examining the level of PacC activation. For example, *ipnA* and *acvA* are divergently transcribed from a common regulatory sequence. One may use constructs (e.g., pAXB4A; Brakhage et al., *supra*) that contain *ipnA-lacZ* and *acvA-uidA* reporters within the same plasmid; this particular plasmid can be targeted to the *argB* locus to ensure integration at a specific genomic locus. A

strain carrying both *argB* and *pyrG* mutations can be sequentially transformed with the *pyrG* and reporter vectors, and enzyme assays can be performed on extracts from mycelia (van Gorcom et al., *Gene*, 1985, 40:99-106; Pobjecky et al., *Mol. Gen. Genet.*, 1990, 220:314-316). In addition, bioassays can be done to determine whether chimeric transcription factors increase the production of fungal secondary metabolites such as penicillin. Supernatant fluid from fermentations can be centrifuged and applied to wells containing indicator organisms such as *Bacillus calidolactis* (Smith el al., *Mol. Gen. Genet.*, 1989, 216:492-497). The application of all of these methods will promote a rapid and quantitative analysis of the efficacy of chimeric transcription factors.

Enhancement of Secondary Metabolite Production

The constructs and methods described herein may be used to increase the yields of currently marketed pharmaceuticals whose production, in whole or in part, is dependent upon a fungal fermentation. For example, in *Aspergillus nidulans*, penicillin biosynthesis is catalyzed by three enzymes encoded by *ipnA*, *acvA*, and *aatA*. Two of these genes, *ipnA* and *acvA*, are regulated directly by PacC. For example, P_{ipnA} contains at least three PacC binding sites (ipnA2, ipnA3, and ipnA4AB)(Espeso and Penalva, *J. Biol. Chem.*, 1996, 271:28825-28830). Expression of a truncated form of PacC has been shown to increase both expression of *ipnA* and *acvA* as well as production of penicillin. Activation (i.e., proteolytoc cleavage) of PacC requires the proteins encoded by the *palA*, *palB*, *palC*, *palF*, *palH*, and *palI* genes. It is possible that increased expression of at least some of these genes would result in increased production of penicillin. In the example described herein, *ipnA* and *acvA* expression are targeted for increase by formation of a chimeric transcription factor including the DNA-binding domain of PacC and 4 VP16 acidic TADs and a proline-rich

TAD. Using the methods of the invention, production of other secondary metabolites can also be increased.

Examples of marketed secondary metabolites whose yields during fermentation could be increased by the methods of the invention include, without limitation, cyclosporin, penicillin, cephalosporin, ergot alkaloids, lovastatin, mevastatin, and the biosynthetic intermediates thereof. In addition, such methods can also be used to increase the likelihood of identifying new secondary metabolites with medicinal or agricultural value by increasing the concentration of such metabolites (and hence, the likelihood of detection by chemical or bioassay) in a fermentation broth.

Production and Detection Methods for Fungal Secondary Metabolites

Methods for fermentation and production of beta-lactam antibiotics, statins, ergot alkaloids, cyclosporin, and other fungal metabolites are described in Masurekar (*Biotechnology*, 1992, 21: 241-301), and references therein. The detection of secondary metabolites is specific for each metabolite and well-known to those practiced in the art. General methods to assess production and integrity of compounds in fermentation broths include, but are not limited to, bioassays for antimicrobial activity, high-performance liquid chromatography (HPLC) analysis, nuclear magnetic resonance, thin-layer chromatography, and absorbance spectrometry. Purification of metabolites from a fermentation broth can include removal of fungal cells or hyphae by centrifugation or filtration, adjustment of pH and/or salt concentrations after fermentation (to enhance solubility and/or subsequent extraction efficiency), and extraction of broths with appropriate organic solvents.

What is claimed is:

1. A chimeric transcription factor comprising

(a) a pre-activated transcription factor functional in a fungal strain, and

(b) a transcription activation domain that is different from the transcription activation domain naturally associated with said transcription factor.

2. The chimeric transcription factor of claim 1, wherein said chimeric transcription factor activates transcription in a manner greater than said pre-activated transcription factor.

3. The chimeric transcription factor of claim 1, wherein said pre-activated transcription factor is pre-activated by truncation.

4. The chimeric transcription factor of claim 1, wherein said pre-activated transcription factor comprises a substitution of a serine or threonine residue with an alanine, aspartic acid, or glutamic acid residue, wherein said substitution pre-activates said transcription factor.

5. The chimeric transcription factor of claim 3, wherein said pre-activated transcription factor is substantially identical to *Aspergillus nidulans* PacC.

6. A chimeric transcription factor comprising

(a) a transcription factor substantially identical to *Aspergillus nidulans* PacC, and

(b) a transcription activation domain that is different from the transcription activation domain naturally associated with said transcription factor.

7. The chimeric transcription factor of claim 1, wherein said pre-activated transcription factor comprises amino acid sequence shown in Fig. 1 (SEQ ID Nos: 1-6).

8. The chimeric transcription factor of claim 1, wherein said pre-activated transcription factor binds to a DNA sequence comprising 5'-GCCAAG-3' or 5'-GCCAGG-3'.

9. A vector comprising DNA encoding the chimeric transcription factor of claim 1 operably linked to a promoter capable of controlling expression of said chimeric transcription factor in a fungal strain.

10. A fungal cell that contains and expresses the DNA of claim 9.

11. The fungal cell of claim 10, wherein said fungal cell is a filamentous fungal cell.

12. The fungal cell of claim 10, wherein said cell produces a secondary metabolite and wherein expression of said DNA increases the production of said secondary metabolite by said cell.

13. The fungal cell of claim 12, wherein said secondary metabolite is non-proteinaceous.

14. The fungal cell of claim 12, wherein said secondary metabolite is a protein or peptide.

15. A method of producing a secondary metabolite, said method comprising culturing the fungal cell of claim 10 under secondary metabolite-producing conditions.

16. A method of producing a secondary metabolite, said method comprising the steps of

(a) introducing into a fungal cell a vector comprising (i) a promoter capable of controlling gene expression in said fungal cell, and (ii) a nucleic acid encoding a transcription factor comprising (i) a DNA-binding domain and (ii) a transcription activation domain; and

(b) culturing said fungal cell under secondary metabolite-producing conditions.

17. The method of claim 16, wherein said fungal cell is a filamentous fungal cell.

18. The method of claim 16, wherein said transcription factor is a chimeric transcription factor.

19. The method of claim 16, wherein said transcription factor is a pre-activated transcription factor.

20. The method of claim 16, wherein said transcription factor is pre-activated by substitution of a serine or threonine residue with an alanine,

aspartic acid, or glutamic acid residue, wherein said substitution pre-activates said transcription factor.

21. The method of claim 16, wherein said transcription factor is pre-activated by truncation.

22. The method of claim 16, wherein said DNA binding domain is from a fungal transcriptional activator.

23. The method of claim 16, wherein said DNA binding domain is from a fungal transcriptional repressor.

24. The method of claim 16, wherein said transcription activation domain that is different from the transcription activation domain naturally associated with said transcription factor.

Figure 1
Sheet 1 of 2

Figure 1
Sheet 2 of 2

SEQUENCE LISTING

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Cys Thr Glu Lys Ser Pro Thr Ala Glu Ser Leu Tyr Glu His Val Cys
65                  70                  75                  80
Glu Arg His Val Gly Arg Lys Ser Thr Asn Asn Leu Asn Leu Thr Cys
                85                  90                  95
Gln Trp Gly Thr Cys Asn Thr Thr Thr Val Lys Arg Asp His Ile Thr
            100                 105                 110
Ser His Ile Arg Val His Val Pro Leu Lys Pro His Lys Cys Asp Phe
        115                 120                 125
Cys Gly Lys Ala Phe Lys Arg Pro Gln Asp Leu Lys Lys His Val Lys
    130                 135                 140
Thr His Ala Asp Asp Ser Glu Ile Arg Ser Pro Glu Pro Gly Met Lys
145                 150                 155                 160
His Pro Asp Met Met Phe Pro Gln Asn Pro Arg Gly Ser Pro Ala Ala
                165                 170                 175
Thr His Tyr Phe Glu Ser Pro Ile Asn Gly Ile Asn Gly Gln Tyr Ser
            180                 185                 190
His Ala Pro Pro Pro Gln Tyr Tyr Gln Pro His Pro Pro Pro Gln Ala
        195                 200                 205
Pro Asn Pro His Ser Tyr Gly Asn Leu Tyr Tyr Ala Leu Ser Gln Gly
    210                 215                 220
Gln Glu Gly Gly His Pro Tyr Asp Arg Lys Arg Gly Tyr Asp Ala Leu
225                 230                 235                 240
Asn Glu Phe Phe Gly Asp Leu Lys Arg Arg Gln Phe Asp Pro Asn Ser
                245                 250                 255
Tyr Ala Ala Val Gly Gln Arg Leu Leu Gly Leu Gln Ala Leu Gln Leu
            260                 265                 270
Pro Phe Leu Ser Gly Pro Ala Pro Glu Tyr Gln Gln Met Pro Ala Pro
        275                 280                 285
Val Ala Val Gly Gly Gly Gly Gly Gly Tyr Gly Gly Gly Ala Pro Gln
    290                 295                 300
Pro Pro Gly Tyr His Leu Pro Pro Met Ser Asn Val Arg Thr Lys Asn
305                 310                 315                 320
Asp Leu Ile Asn Ile Asp Gln Phe Leu Glu Gln Met Gln Asn Thr Ile
                325                 330                 335
Tyr Glu Ser Asp Glu Asn Val Ala Ala Ala Gly Val Ala Gln Pro Gly
            340                 345                 350
Ala His Tyr Val His Gly Gly Met Asn His Arg Thr Thr His Ser Pro
        355                 360                 365
Pro Thr His Ser Arg Gln Ala Thr Leu Leu Gln Leu Pro Ser Ala Pro
    370                 375                 380
Met Ala Ala Ala Thr Ala His Ser Pro Ser Val Gly Thr Pro Ala Leu
385                 390                 395                 400
Thr Pro Pro Ser Ser Ala Gln Ser Tyr Thr Ser Asn Arg Ser Pro Ile
                405                 410                 415
Ser Leu His Ser Ser Arg Val Ser Pro Pro His Glu Glu Ala Ala Pro
            420                 425                 430
Gly Met Tyr Pro Arg Leu Pro Ala Ala Ile Cys Ala Asp Ser Met Thr
        435                 440                 445
Ala Gly Tyr Pro Thr Ala Ser Gly Ala Ala Pro Pro Ser Thr Leu Ser
    450                 455                 460
Gly Ala Tyr Asp His Asp Asp Arg Arg Arg Tyr Thr Gly Gly Thr Leu
465                 470                 475                 480
Gln Arg Ala Arg Pro Ala Glu Arg Ala Ala Thr Glu Asp Arg Met Asp
                485                 490                 495
```

```
Ile Ser Gln Asp Ser Lys His Asp Gly Glu Arg Thr Pro Lys Ala Met
            500                 505                 510
His Ile Ser Ala Ser Leu Ile Asp Pro Ala Leu Ser Gly Thr Ser Ser
        515                 520                 525
Asp Pro Glu Gln Glu Ser Ala Lys Arg Thr Ala Ala Thr Ala Thr Glu
    530                 535                 540
Val Ala Glu Arg Asp Val Asn Val Ala Trp Val Glu Lys Val Arg Leu
545                 550                 555                 560
Leu Glu Asn Leu Arg Arg Leu Val Ser Gly Leu Leu Glu Ala Gly Ser
                565                 570                 575
Leu Thr Pro Glu Tyr Gly Val Gln Thr Ser Ser Ala Ser Pro Thr Pro
            580                 585                 590
Gly Leu Asp Ala Met Glu Gly Val Glu Thr Ala Ser Val Arg Ala Ala
        595                 600                 605
Ser Glu Gln Ala Arg Glu Glu Pro Lys Ser Glu Ser Glu Gly Val Phe
    610                 615                 620
Tyr Pro Thr Leu Arg Gly Val Asp Glu Asp Glu Asp Gly Asp Ser Lys
625                 630                 635                 640
Met Pro Glu
```

<210> 4
<211> 585
<212> PRT
<213> Yarrowia lipolytica

<400> 4

```
Met Ala Ser Tyr Pro Tyr Leu Ala Gln Ser Gln Pro Pro Gln Gln Gln
 1               5                  10                  15
Gln Gln Gln Gln Gln Gln Pro Gln Gln Gln Ser Gln Gln Leu Pro Thr
            20                  25                  30
Thr Ala Pro Ser Ala Ala Pro Gln Val Asn Asn Thr Thr Ala Asn Lys
        35                  40                  45
Pro Leu Tyr Pro Ala Ser Pro Asn Ser Pro Ile Ser Pro Ser Asp Tyr
    50                  55                  60
Ser Ala Asn Met Asn Val Gly Gly Asp Ser Val Asp Met Leu Leu Ser
65                  70                  75                  80
Ser Val Ser Ala His His Arg Ser Ser Asp Ala Gly Gln Ser Asp Met
                85                  90                  95
Gly Ser Ile Ser Pro Ser Thr Ala His Thr Thr Pro Asp Ala Thr Thr
            100                 105                 110
Tyr Lys Thr Ser Asp Glu Glu Asp Ala Thr Gly Lys Ile Thr Thr Pro
        115                 120                 125
Arg Ser Glu Gly Ser Pro Asn Thr Asn Gly Ser Gly Ser Asp Gly Glu
    130                 135                 140
Asn Leu Val Cys Lys Trp Gly Pro Cys Gly Lys Thr Phe Gly Ser Ala
145                 150                 155                 160
Glu Lys Leu Tyr Ala His Leu Cys Asp Ala His Val Gly Arg Lys Cys
                165                 170                 175
Thr His Asn Leu Ser Leu Val Cys Asn Trp Asp Asn Cys Gly Ile Val
            180                 185                 190
Thr Val Lys Arg Asp His Ile Thr Ser His Ile Arg Val His Val Pro
        195                 200                 205
Leu Lys Pro Tyr Lys Cys Asp Phe Cys Thr Lys Ser Phe Lys Arg Pro
    210                 215                 220
```

Gln Asp Leu Lys Lys His Val Lys Thr His Ala Asp Asp Asn Glu Gln
225 230 235 240
Ala His Asn Ala Tyr Ala Lys Pro His Met Gln His Thr His Gln Gln
245 250 255
Gln Gln Gln Gln Gln Arg Tyr Met Gln Tyr Pro Thr Tyr Ala Ser Gly
260 265 270
Tyr Glu Tyr Pro Tyr Tyr Arg Tyr Ser Gln Pro Gln Val Gln Val Pro
275 280 285
Met Val Pro Ser Tyr Ala Ala Val Gly His Met Pro Thr Pro Pro Met
290 295 300
His Pro His Ala Pro Ile Asp Arg Lys Arg Gln Trp Asp Thr Thr Ser
305 310 315 320
Asp Phe Phe Asp Asp Ile Lys Arg Ala Arg Val Thr Pro Asn Tyr Ser
325 330 335
Ser Asp Ile Ala Ser Arg Leu Ser Thr Ile Glu Gln Tyr Ile Gly Ile
340 345 350
Gln Gly Gln Gln Gln Gln Ala Ser Pro Thr Pro Gln Thr Ala Thr Thr
355 360 365
Thr Ser Ala Thr Pro Ala Pro Ala Ala Pro His Gln Ala Thr Pro Pro
370 375 380
Gln Gln Gln Leu Pro Ser Phe Lys Gln Gly Asp Tyr Gln Glu Thr Asp
385 390 395 400
Gln Phe Leu Asn Gln Leu Gly Ser Asn Ile Tyr Gly Asn Ile Lys Ser
405 410 415
Val Asp Pro Gln Tyr Glu Ala Pro Ala Glu Phe His Leu Pro His Pro
420 425 430
Met Gly Tyr Arg Tyr Ala Phe Ser His Ala Pro Ala Pro His Gly Ala
435 440 445
Ala Pro Val Ala Pro Gln Val Ala Pro Pro Ala His Pro Gly Val His
450 455 460
Gly Val Ser Ala Pro His Tyr Pro Asp Leu Ser Tyr Ser Arg Ser Thr
465 470 475 480
Val Pro Gln Leu Ser Ser Arg Phe Glu Asp Val Arg Gln Met Ser Val
485 490 495
Gly Val Thr Gln Arg Ala Ala Arg Thr Thr Asn Val Glu Glu Ser Asp
500 505 510
Asp Asp Asp Glu Leu Val Glu Gly Phe Gly Lys Met Ala Ile Ala Asp
515 520 525
Ser Lys Ala Met Gln Val Ala Gln Met Lys Lys His Leu Glu Val Val
530 535 540
Ser Tyr Leu Arg Arg Val Leu Gln Glu Ala Arg Glu Thr Glu Ser Gly
545 550 555 560
Glu Ala Glu Asp Thr Ala Ala Asn Lys Asp Thr Ser Ala Ser Lys Ser
565 570 575
Ser Leu Tyr Pro Thr Ile Lys Ala Cys
580 585

<210> 5
<211> 659
<212> PRT
<213> Candida albicans

<400> 5
Met Asn Tyr Asn Ile His Pro Val Thr Tyr Leu Asn Ala Asp Ser Asn
1 5 10 15

```
Thr Gly Ala Ser Glu Ser Thr Ala Ser His His Gly Ser Lys Lys Ser
                20                  25                  30
Pro Ser Ser Asp Ile Asp Val Asp Asn Ala Xaa Ser Pro Ser Ser Phe
            35                  40                  45
Thr Ser Ser Gln Ser Pro His Ile Asn Ala Met Gly Asn Ser Pro His
        50                  55                  60
Ser Ser Phe Thr Ser Gln Ser Ala Ala Asn Ser Pro Ile Thr Asp Ala
65                  70                  75                  80
Lys Gln His Leu Val Lys Pro Thr Thr Thr Lys Pro Ala Ala Phe Ala
                85                  90                  95
Pro Ser Ala Asn Gln Ser Asn Thr Thr Ala Pro Gln Ser Tyr Thr Gln
            100                 105                 110
Pro Ala Gln Gln Leu Pro Thr Gln Leu His Pro Ser Leu Asn Gln Ala
        115                 120                 125
Tyr Asn Asn Gln Pro Ser Tyr Tyr Leu His Gln Pro Thr Tyr Gly Tyr
    130                 135                 140
Gln Gln Gln Gln Gln Gln Gln Gln His Gln Glu Phe Asn Gln Pro Ser
145                 150                 155                 160
Gln Gln Tyr His Asp His His Gly Tyr Tyr Ser Asn Asn Asn Ile Leu
                165                 170                 175
Asn Gln Asn Gln Pro Ala Pro Gln Gln Asn Pro Val Lys Pro Phe Lys
            180                 185                 190
Lys Thr Tyr Lys Lys Ile Arg Asp Glu Asp Leu Lys Gly Pro Phe Lys
        195                 200                 205
Cys Leu Trp Ser Asn Cys Ser Ile Ile Phe Glu Thr Pro Glu Ile Leu
    210                 215                 220
Tyr Asp His Leu Cys Asp Asp His Val Gly Arg Lys Ser Ser Asn Asn
225                 230                 235                 240
Leu Ser Leu Thr Cys Leu Trp Glu Asn Cys Gly Thr Thr Thr Val Lys
                245                 250                 255
Arg Asp His Ile Thr Ser His Leu Arg Val His Val Pro Leu Lys Pro
            260                 265                 270
Phe His Cys Asp Leu Cys Pro Lys Ser Phe Lys Arg Pro Gln Asp Leu
        275                 280                 285
Lys Lys His Ser Lys Thr His Ala Glu Asp His Pro Lys Lys Leu Lys
    290                 295                 300
Lys Ala Gln Arg Glu Leu Met Lys Gln Gln Gln Lys Glu Ala Lys Gln
305                 310                 315                 320
Gln Gln Lys Leu Ala Asn Lys Arg Ala Asn Ser Met Asn Ala Thr Thr
                325                 330                 335
Ala Ser Asp Leu Gln Leu Asn Tyr Tyr Ser Gly Asn Pro Ala Asp Gly
            340                 345                 350
Leu Asn Tyr Asp Asp Thr Ser Lys Lys Arg Arg Tyr Glu Asn Asn Ser
        355                 360                 365
Gln His Asn Met Tyr Val Val Asn Ser Ile Leu Asn Asp Phe Asn Phe
    370                 375                 380
Gln Gln Met Ala Gln Ala Pro Gln Gln Pro Gly Val Val Gly Thr Ala
385                 390                 395                 400
Gly Ser Ala Glu Phe Thr Thr Lys Arg Met Lys Ala Gly Thr Glu Tyr
                405                 410                 415
Asn Ile Asp Val Phe Asn Lys Leu Asn His Leu Asp Asp His Leu His
            420                 425                 430
His His His Pro Gln Gln Gln His Pro Gln Gln Gln Tyr Gly Gly Asn
        435                 440                 445
Ile Tyr Glu Ala Glu Lys Phe Phe Asn Ser Leu Ser Asn Ser Ile Asp
```

```
    450                 455                 460
Met Gln Tyr Gln Asn Met Ser Thr Gln Tyr Gln Gln Gln His Ala Gly
465                 470                 475                 480
Ser Thr Phe Ala Gln Gln Lys Pro Thr Gln Gln Ala Ser Gly Gln Leu
                485                 490                 495
Tyr Pro Ser Leu Pro Thr Ile Gly Asn Gly Ser Tyr Thr Ser Gly Ser
            500                 505                 510
Ser His Lys Glu Gly Leu Val Asn Asn His Asn Gly Tyr Leu Pro Ser
        515                 520                 525
Tyr Pro Gln Ile Asn Arg Ser Leu Pro Tyr Ser Ser Gly Val Ala Gln
    530                 535                 540
Gln Pro Pro Ser Ala Leu Glu Phe Gly Gly Val Ser Thr Tyr Gln Lys
545                 550                 555                 560
Ser Ala Gln Ser Tyr Glu Glu Asp Ser Ser Asp Ser Ser Glu Glu Asp
                565                 570                 575
Asp Tyr Ser Thr Ser Ser Glu Asp Glu Leu Asp Thr Leu Phe Asp Lys
            580                 585                 590
Leu Asn Ile Asp Asp Asn Lys Val Glu Glu Val Thr Ile Asp Gly Phe
        595                 600                 605
Asn Leu Lys Asp Val Ala Lys His Arg Glu Met Ile His Ala Val Leu
    610                 615                 620
Gly Tyr Leu Arg Asn Gln Ile Glu Gln Gln Glu Lys Glu Lys Ser Lys
625                 630                 635                 640
Glu Gln Lys Glu Val Asp Val Asn Glu Thr Lys Leu Tyr Pro Thr Ile
                645                 650                 655
Thr Ala Phe
```

<210> 6
<211> 625
<212> PRT
<213> Saccharomyces cerevisiae

<400> 6

```
Met Val Pro Leu Glu Asp Leu Leu Asn Lys Glu Asn Gly Thr Ala Ala
 1               5                  10                  15
Pro Gln His Ser Arg Glu Ser Ile Val Glu Asn Gly Thr Asp Val Ser
            20                  25                  30
Asn Val Thr Lys Lys Asp Gly Leu Pro Ser Pro Asn Leu Ser Lys Arg
        35                  40                  45
Ser Ser Asp Cys Ser Lys Arg Pro Arg Ile Arg Cys Thr Thr Glu Ala
    50                  55                  60
Ile Gly Leu Asn Gly Gln Glu Asp Glu Arg Met Ser Pro Gly Ser Thr
65                  70                  75                  80
Ser Ser Ser Cys Leu Pro Tyr His Ser Thr Ser His Leu Asn Thr Pro
                85                  90                  95
Pro Tyr Asp Leu Leu Gly Ala Ser Ala Val Ser Pro Thr Thr Ser Ser
            100                 105                 110
Ser Ser Asp Ser Ser Ser Ser Ser Pro Leu Ala Gln Ala His Asn Pro
        115                 120                 125
Ala Gly Asp Asp Asp Asp Ala Asp Asn Asp Gly Asp Ser Glu Asp Ile
    130                 135                 140
Thr Leu Tyr Cys Lys Trp Asp Asn Cys Gly Met Ile Phe Asn Gln Pro
145                 150                 155                 160
Glu Leu Leu Tyr Asn His Leu Cys His Asp His Val Gly Arg Lys Ser
```

```
                    165                 170                 175
His Lys Asn Leu Gln Leu Asn Cys His Trp Gly Asp Cys Thr Thr Lys
                180                 185                 190
Thr Glu Lys Arg Asp His Ile Thr Ser His Leu Arg Val His Val Pro
            195                 200                 205
Leu Lys Pro Phe Gly Cys Ser Thr Cys Ser Lys Lys Phe Lys Arg Pro
        210                 215                 220
Gln Asp Leu Lys Lys His Leu Lys Ile His Leu Glu Ser Gly Gly Ile
225                 230                 235                 240
Leu Lys Arg Lys Arg Gly Pro Lys Trp Gly Ser Lys Arg Thr Ser Lys
                245                 250                 255
Lys Asn Lys Ser Cys Ala Ser Asp Ala Val Ser Ser Cys Ser Ala Ser
            260                 265                 270
Val Pro Ser Ala Ile Ala Gly Ser Phe Lys Ser His Ser Thr Ser Pro
        275                 280                 285
Gln Ile Leu Pro Pro Leu Pro Val Gly Ile Ser Gln His Leu Pro Ser
    290                 295                 300
Gln Gln Gln Gln Arg Ala Ile Ser Leu Asn Gln Leu Cys Ser Asp Glu
305                 310                 315                 320
Leu Ser Gln Tyr Lys Pro Val Tyr Ser Pro Gln Leu Ser Ala Arg Leu
                325                 330                 335
Gln Thr Ile Leu Pro Pro Leu Tyr Tyr Asn Asn Gly Ser Thr Val Ser
            340                 345                 350
Gln Gly Ala Asn Ser Arg Ser Met Asn Val Tyr Glu Asp Gly Cys Ser
        355                 360                 365
Asn Lys Thr Ile Ala Asn Ala Thr Gln Phe Phe Thr Lys Leu Ser Arg
    370                 375                 380
Asn Met Thr Asn Asn Tyr Ile Leu Gln Gln Ser Gly Gly Ser Thr Glu
385                 390                 395                 400
Ser Ser Ser Ser Ser Gly Arg Ile Pro Val Ala Gln Thr Ser Tyr Val
                405                 410                 415
Gln Pro Pro Asn Ala Pro Ser Tyr Gln Ser Val Gln Gly Gly Ser Ser
            420                 425                 430
Ile Ser Ala Thr Ala Asn Thr Ala Thr Tyr Val Pro Val Arg Leu Ala
        435                 440                 445
Lys Tyr Pro Thr Gly Pro Ser Leu Thr Glu His Leu Pro Pro Leu His
    450                 455                 460
Ser Asn Thr Ala Gly Gly Val Phe Asn Arg Gln Ser Gln Tyr Ala Met
465                 470                 475                 480
Pro His Tyr Pro Ser Val Arg Ala Ala Pro Ser Tyr Ser Ser Ser Gly
                485                 490                 495
Cys Ser Ile Leu Pro Pro Leu Gln Ser Lys Ile Pro Met Leu Pro Ser
            500                 505                 510
Arg Arg Thr Met Ala Gly Gly Thr Ser Leu Lys Pro Asn Trp Glu Phe
        515                 520                 525
Ser Leu Asn Gln Lys Ser Cys Thr Asn Asp Ile Ile Met Ser Lys Leu
    530                 535                 540
Ala Ile Glu Glu Val Asp Asp Glu Ser Glu Ile Glu Asp Asp Phe Val
545                 550                 555                 560
Glu Met Leu Gly Ile Val Asn Ile Ile Lys Asp Tyr Leu Leu Cys Cys
                565                 570                 575
Val Met Glu Asp Leu Asp Asp Glu Glu Ser Glu Asp Lys Asp Glu Glu
            580                 585                 590
Asn Ala Phe Leu Gln Glu Ser Leu Glu Lys Leu Ser Leu Gln Asn Gln
        595                 600                 605
```

Met Gly Thr Asn Ser Val Arg Ile Leu Thr Lys Tyr Pro Lys Ile Leu
610 615 620
Val
625

<210> 7
<211> 815
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic primer based on Aspergillus nidulans and herpes virus

<400> 7
Met Ser Ser Arg Gly Ala Met Ala Glu Glu Ala Val Ala Pro Val Ala
1 5 10 15
Val Pro Thr Thr Gln Glu Gln Pro Thr Ser Gln Pro Ala Ala Ala Gln
20 25 30
Val Thr Thr Val Thr Ser Pro Ser Val Thr Ala Thr Ala Ala Ala Ala
35 40 45
Thr Ala Ala Val Ala Ser Pro Gln Ala Asn Gly Asn Ala Ala Ser Pro
50 55 60
Val Ala Pro Ala Ser Ser Thr Ser Arg Pro Ala Glu Glu Leu Thr Cys
65 70 75 80
Met Trp Gln Gly Cys Ser Glu Lys Leu Pro Thr Pro Glu Ser Leu Tyr
85 90 95
Glu His Val Cys Glu Arg His Val Gly Arg Lys Ser Thr Asn Asn Leu
100 105 110
Asn Leu Thr Cys Gln Trp Gly Ser Cys Arg Thr Thr Thr Val Lys Arg
115 120 125
Asp His Ile Thr Ser His Ile Arg Val His Val Pro Leu Lys Pro His
130 135 140
Lys Cys Asp Phe Cys Gly Lys Ala Phe Lys Arg Pro Gln Asp Leu Lys
145 150 155 160
Lys His Val Lys Thr His Ala Asp Asp Ser Val Leu Val Arg Ser Pro
165 170 175
Glu Pro Gly Ser Arg Asn Pro Asp Met Met Phe Gly Gly Asn Gly Lys
180 185 190
Gly Tyr Ala Ala Ala His Tyr Phe Glu Pro Ala Leu Asn Pro Val Pro
195 200 205
Ser Gln Gly Tyr Ala His Gly Pro Pro Gln Tyr Tyr Gln Ala His His
210 215 220
Ala Pro Gln Pro Ser Asn Pro Ser Tyr Gly Asn Val Tyr Tyr Ala Leu
225 230 235 240
Asn Thr Gly Pro Glu Pro His Gln Ala Ser Tyr Glu Ser Lys Lys Arg
245 250 255
Gly Tyr Asp Ala Leu Asn Glu Phe Phe Gly Asp Leu Lys Arg Arg Gln
260 265 270
Phe Asp Pro Asn Ser Tyr Ala Ala Val Gly Gln Arg Leu Leu Ser Leu
275 280 285
Gln Asn Leu Ser Leu Pro Val Leu Thr Ala Ala Pro Leu Pro Glu Tyr
290 295 300
Gln Ala Met Pro Ala Pro Val Ala Val Ala Ser Gly Pro Tyr Gly Gly
305 310 315 320

```
Gly Pro His Pro Ala Pro Ala Tyr His Leu Pro Pro Met Ser Asn Val
                325                 330                 335
Arg Thr Lys Asn Asp Leu Ile Asn Ile Asp Gln Phe Leu Gln Gln Met
            340                 345                 350
Gln Asp Thr Ile Tyr Glu Asn Asp Asp Asn Val Ala Ala Ala Gly Val
        355                 360                 365
Ala Gln Pro Gly Ala His Tyr Ile His Asn Gly Ile Ser Tyr Arg Thr
    370                 375                 380
Thr His Ser Pro Pro Thr Gln Leu Pro Ser Ala His Ala Thr Thr Gln
385                 390                 395                 400
Thr Thr Ala Gly Pro Ile Ile Ser Asn Thr Ser Ala His Ser Pro Ser
                405                 410                 415
Ser Ser Thr Pro Ala Leu Thr Pro Pro Ser Ser Ala Gln Ser Tyr Thr
            420                 425                 430
Ser Gly Arg Ser Pro Ile Ser Leu Pro Ser Ala His Arg Val Ser Pro
        435                 440                 445
Pro His Glu Ser Gly Ser Ser Met Tyr Pro Arg Leu Pro Ser Ala Thr
    450                 455                 460
Asp Gly Met Thr Ser Gly Tyr Gly Ser Pro Pro Pro Pro Pro Pro Pro
465                 470                 475                 480
Pro Pro Pro Pro Ile Lys Val Ala Pro Pro Thr Asp Val Ser Leu Gly
                485                 490                 495
Asp Glu Leu His Leu Asp Gly Glu Asp Val Ala Met Ala His Ala Asp
            500                 505                 510
Ala Leu Asp Asp Phe Asp Leu Asp Met Leu Gly Asp Gly Asp Ser Pro
        515                 520                 525
Gly Pro Gly Phe Thr Pro His Asp Ser Ala Pro Tyr Gly Ala Leu Asp
    530                 535                 540
Met Ala Asp Phe Glu Phe Glu Gln Met Phe Thr Asp Ala Leu Gly Ile
545                 550                 555                 560
Asp Glu Tyr Gly Gly Asp Ile Lys Val Ala Pro Pro Thr Asp Val Ser
                565                 570                 575
Leu Gly Asp Glu Leu His Leu Asp Gly Glu Asp Val Ala Met Ala His
            580                 585                 590
Ala Asp Ala Leu Asp Asp Phe Asp Leu Asp Met Leu Gly Asp Gly Asp
        595                 600                 605
Ser Pro Gly Pro Gly Phe Thr Pro His Asp Ser Ala Pro Tyr Gly Ala
    610                 615                 620
Leu Asp Met Ala Asp Phe Glu Phe Glu Gln Met Phe Thr Asp Ala Leu
625                 630                 635                 640
Gly Ile Asp Glu Tyr Gly Gly Asp Ile Lys Val Ala Pro Pro Thr Asp
                645                 650                 655
Val Ser Leu Gly Asp Glu Leu His Leu Asp Gly Glu Asp Val Ala Met
            660                 665                 670
Ala His Ala Asp Ala Leu Asp Asp Phe Asp Leu Asp Met Leu Gly Asp
        675                 680                 685
Gly Asp Ser Pro Gly Pro Gly Phe Thr Pro His Asp Ser Ala Pro Tyr
    690                 695                 700
Gly Ala Leu Asp Met Ala Asp Phe Glu Phe Glu Gln Met Phe Thr Asp
705                 710                 715                 720
Ala Leu Gly Ile Asp Glu Tyr Gly Gly Asp Ile Lys Val Ala Pro Pro
                725                 730                 735
Thr Asp Val Ser Leu Gly Asp Glu Leu His Leu Asp Gly Glu Asp Val
            740                 745                 750
Ala Met Ala His Ala Asp Ala Leu Asp Asp Phe Asp Leu Asp Met Leu
```

```
        755                 760                 765
Gly Asp Gly Asp Ser Pro Gly Pro Gly Phe Thr Pro His Asp Ser Ala
    770                 775                 780
Pro Tyr Gly Ala Leu Asp Met Ala Asp Phe Glu Phe Glu Gln Met Phe
785                 790                 795                 800
Thr Asp Ala Leu Gly Ile Asp Glu Tyr Gly Gly Asp Gly Leu Gln
                805                 810                 815
```

<210> 8
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer based on Aspergillus nidulans

<400> 8
aactgcagta gttgaccgtg tgattgggtt ct 32

<210> 9
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer based on Aspergillus nidulans

<400> 9
ccggaattct ttgtaaactg gcttgaagat 30

<210> 10
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic oligonucleotide encoding proline rich motif

<400> 10
gatccccccc ccctcctcca cccccacccc ctccc 35

<210> 11
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic oligonucleotide encoding proline rich motif

<400> 11
gggagggggt gggggtggag gagggggggg g 31

<210> 12
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer based on herpes simplex virus

<400> 12
cgcgatatca aagtcgcccc cccgaccgat 30

<210> 13
<211> 30
<212> DNA
<213> Synthetic primer based on Aspergillus nidulans

<400> 13
cgcgatatcc ccaccgtact cgtcaattcc 30

<210> 14
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer based on Aspergillus nidulans

<400> 14
tgctctagag gcgccatggc cgaagaagcg 30

<210> 15
<211> 30
<212> DNA
<213> Aspergillus nidulans

<400> 15
cgcggatccg taaccagaag tcataccgtc 30

<210> 16
<211> 1413
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer based on Aspergillus nidulans

<400> 16
tctagaggcg ccatggccga agaagcggtc gctcctgtag ctgtgcctac gacccaagaa 60
caaccaacct ctcaacccgc cgctgcgcag gttacaactg tcacttcgcc ctctgtgact 120
gcaacagcgg cggctgcgac agctgctgtg gccagtcccc aagctaatgg caatgctgcc 180
tctcctgtcg cccctgcgtc gtcaacatct cgtccagcgg aagaactcac ttgcatgtgg 240
caaggctgct ctgagaagct ccctactcca gaatccttat acgaacatgt ctgcgagcgt 300
cacgttggcc gaaagagcac gaacaacctc aacctgactt gtcaatgggg tagctgtcgt 360
actactactg tgaaacgcga ccatatcacc tctcatatcc gggtgcacgt tcctctcaag 420
ccgcacaagt gtgatttctg tggaaaagcg ttcaagcgtc cccaggattt gaagaagcat 480

gttaagacgc acgctgatga ctcggtcctg gtacggtcgc cagagcctgg atctcgcaac 540
ccagatatga tgttcggagg aaatggcaag ggctatgctg ctgcgcacta ttttgagcct 600
gctctcaacc ctgttcccag ccaaggctac gctcatggtc ctccccagta ttaccaggcc 660
catcacgctc cccagccatc gaacccgtct tacggcaacg tctactacgc tctgaatacc 720
ggcccagagc ctcaccaagc gtcgtatgaa tccaagaagc ggggttatga tgcgcttaat 780
gagttctttg gtgacctcaa gcgccgacaa tttgacccta attcctacgc tgccgtgggc 840
cagcgcctgc tcagtttgca gaacttgtcc ctgcctgttt taacggctgc gcctctgccc 900
gagtaccagg caatgcctgc tcctgtggct gttgctagtg gtccatatgg tggcggccct 960
caccctgcgc cggcatatca tcttccacca atgagcaacg tccgaaccaa gaacgacttg 1020
atcaacatcg accagttcct gcagcaaatg caggacacaa tatatgagaa cgatgataat 1080
gtcgctgcgg ctggtgtcgc tcaacctgga gcccattaca ttcataacgg cataagctac 1140
cgcactacac actcgcctcc gacacaactt ccctcggcac atgccacaac ccagacgact 1200
gctggtccta ttatctcaaa cacatctgcg cactcccctt cgtctagcac tccggctttg 1260
acaccgccct caagtgcgca gtcgtacact tcaggtcgct ctcccatttc acttccgtct 1320
gctcatcgcg tttctccgcc tcatgaaagc ggctccagca tgtaccctcg tctcccttcg 1380
gcgactgacg gtatgacttc tggttacgga tcc 1413

<210> 17
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer based on Aspergillus nidulans

<400> 17
ataagaatgc ggccgccctc tgcattattg tcttatc 37

<210> 18
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer based on Aspergillus nidulans

<400> 18
tgctctagaa gacattgttg ctatagctgt 30

<210> 19
<211> 678
<212> DNA
<213> Aspergillus nidulans

<400> 19
gcggccgccc tctgcattat tgtcttatcc gctattcctg gtgtttttgt tgtcttacta 60
ctttttgtgt cgttgaaatt cttactaggc gttgtgaatc tggatcggat catgctattt 120
tgaggtgtaa tgcatgggtc aaatttttctc gagtttcaaa cgaggcagaa gagagatgca 180
gataaatctt gagttttatc atgcagcgaa cgttaccact tatagtttcc ggcagagcac 240
gtaggtcggc ccggcgtcat gtgtagcggg ggagctccag gaccttgagg acgaaaatgg 300
gacggcgatg tataactcca tggaggaacg gagcgtgatt ttgtactgtc tgatccgagg 360
ctaatgagaa agcggaggtt caatgttccc ccggttgatg tcctgaagca gcgaggcccg 420
aagtatcccg tcgtggacat gacatcagtg gtccgactcc cgccgaaccc tcctccttca 480
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/24975

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C07K 14/00; C12N 1/15, 15/63; C12P 21/02
US CL :435/69.1, 254.11, 320.1; 530/300, 350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 254.11, 320.1; 530/300, 350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,589,362 A (BUJARD et al) 31 December 1996, see entire document, especially columns 2-14.	1-24
Y	WANG et al. Positive and negative regulation of gene expression in eukaryotic cells with an inducible transcriptional regulator. Gene Therapy. June 1997, Vol. 4, pages 432-441, see entire document, especially pages 432-433.	1-24
Y	GERBER et al. Transcriptional Activation Modulated by Homopolymeric Glutamine and Proline Stretches. Science. 11 February 1994, Vol. 263, pages 808-811, see entire document, especially page 808.	1-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 10 FEBRUARY 1999	Date of mailing of the international search report 25 FEB 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer TERRY A. MCKELVEY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/24975

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TILBURN et al. The Aspergillus PacC zinc finger transcription factor mediates regulation of both acid- and alkaline- expressed genes by ambient pH. The EMBO Journal. 04 February 1995, Vol. 14, No. 4, pages 779-790, see entire document, especially page 779.	1-24
Y	O'REILLY et al. A single serine residue at position 375 of VP16 is critical for complex assembly with Oct-1 and HCF and is a target of phosphorylation by casein kinase II. The EMBO Journal. 01 May 1997, Vol. 16, No. 9, pages 2420-2430, see entire document, especially page 2420.	1, 4, 16, 20
Y	HAO et al. Mutation of Phosphoserine 389 Affects p53 Function in Vivo. The Journal of Biological Chemistry. 15 November 1996, Vol. 271, No. 46, pages 29380-29385, see entire document, especially page 29380.	1, 4, 16, 20
Y	US 5,462,862 A (GROENEN et al) 31 October 1995, see entire document, especially columns 1-3.	9-24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/24975

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG (biotechnology OneSearch databases):
search terms: chimer?, transcription factor?, activat?, pre-activat?, domain?, truncat?, serine, threonine, alanine, aspartic, glutamic, substitut?, replac?, exchang?, PacC, nidulans, fung?, secondary metabolite?, express?, overexpress?, increas?, enhanc?, penicillin, cephalosporin, lovastatin, compactin, cyclosporin, pneumocandin, echinocandin, DNA binding domain?.